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Putative Immunological Influence Upon Amphibian Forelimb Regeneration. II. Effects of X-Irradiation on Regeneration and Allograft Rejection

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Abstract. Influence of the immune system on epimorphic regeneration of amphibian limbs has been suggested but not proved. The present investigation explored this hypothesis by examining the effects of x-irradiation on forelimb regeneration and rejection of skin allografts. Two kRad x-irradiation was provided either to a single limb or as whole-body irradiation to intact newts (with 1 limb shielded). Complete suppression of regeneration was observed when limbs to be amputated were irradiated directly. In addition, irradiated limbs displayed severe and protracted inflammation, with total resorption of the affected limbs in 85% of the cases. Moreover, delays in both the rate of forelimb regeneration and allograft rejection were found in animals receiving whole-body irradiation. However, in these cases neither forelimb regeneration nor allograft rejection were suppressed. These observations diffuse the challenge raised by irradiation studies to the notion of possible immunological influence on epimorphic regeneration. Moreover, the delays observed in both regeneration rate and allograft rejection following whole-body irradiation are consistent with possible interaction between the immune system and the regenerating limb. Nevertheless, confirmation that such interaction occurs and is integral to epimorphic regeneration must await further investigations.

Introduction

Epimorphic regeneration of lost appendages in vertebrates is accomplished by a mass of dedifferentiated tissues—the regeneration blastema. This mass of tissue is derived at the site of amputation (Butler, 1935; Brunst and Cheremetieva, 1936; Butler and O'Brien, 1942). Although it has been well-established that the blastema is not produced by an accumulation of blood cells, an influence of the immune system on the progress of regeneration has been suggested (Prehn, 1970). This hypothesis arose because of perceived similarities between sarcomas and the regeneration blastema. In essence, this hypothesis considers that immunostimulation might promote blastemal growth just as mild immunostimulation promotes the growth of certain sarcomas (Prehn, 1970, 1972; Prehn and Lappé, 1971).

The validity of this hypothesis has yet to be either confirmed or refuted conclusively. Nevertheless, several studies have demonstrated effects on the progress of regeneration of substances that might have altered immunological status (Sicard, 1981; Schotté and Sicard, 1982; and Sicard and Laffond, 1983). These experiments employed chemical interventions and assessed their impact on forelimb regeneration but not on immunological function. More recent investigations have examined parameters of immunological status (Sicard and Lombard, 1989). One of these studies disclosed modified responsiveness to T-cell mitogens during epimorphic regeneration (diminished responsiveness during dedifferentiation and blastema formation; augmented responsiveness during proliferative stages) but not following nonamputational trauma. Another study observed reciprocal effects of graft rejection and forelimb regeneration. That is, regeneration rate could be accelerated or delayed when combined with allograft challenge, depending upon the temporal relationship between both operations. Similarly, the rate of allograft rejection could be altered by events of epimorphic regeneration.

Although the results of these previous investigations

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are consistent with immunological influence on epimorphic regeneration, they cannot yet prove that such influence occurs. On the other hand, the studies that established that the blastema is of local origin (Butler, 1935; Brunst and Cheremetieva, 1936; Butler and O'Brien, 1942) might present a formidable challenge to this notion. If whole-body irradiation rendered the subjects immunoincompetent, their subsequent regeneration of forelimbs occurred without immunological influence. However, if the animals retained immunocompetence, then regeneration occurred against a background of potential immunological influence. The absence of specific mention of increased morbidity or mortality in irradiated animals by earlier investigators leads us to the conclusion that immunocompetence was at least adequate to meet challenges of infection in these animals. Nevertheless, this requires verification.

The present investigation was undertaken to confront this potential challenge to the hypothesis that the immune system might influence the initiation and progress of epimorphic regeneration. This investigation employed x-irradiation as the means of intervention and assessed its effects both on forelimb regeneration and immune status (skin allograft rejection). Documentation of an impaired immune response (*i.e.*, delayed graft rejection) in the absence of an effect on forelimb regeneration would challenge or refute the immunostimulation hypothesis as it applies to epimorphic regeneration. On the other hand, demonstration of intact immunocompetence (*i.e.*, essentially normal allograft rejection) in irradiated newts regenerating amputated forelimbs removes the challenge presented by these earlier studies. However, it cannot establish that the immune system plays a role in epimorphic regeneration.

Materials and Methods

Animals

Adult newts (*Notophthalmus viridescens*), obtained from Tennessee, were maintained in glass bowls in Holtfreter's solution at 21 ± 2°C. Two control groups were established: (1) regenerating controls and (2) allograft controls. These groups received no treatment other than amputation or a skin graft and marked the "normal" rate of regeneration or graft rejection. Irradiations and surgical manipulations were performed on newts anesthetized in 0.1% aqueous methane tricaine sulfonate (MS-222).

Irradiation

Initial treatment consisted of x-irradiation. Experimental animals were divided into two groups, one receiving whole-body irradiation, except for a shielded limb, and a second group in which only one limb was irradiated. Total irradiation was either 2 kRad (whole-body and limb) or 2.2 kRad (whole body only). Irradiation was provided using a Picker-Gemini 320 kV industrial x-ray unit equipped with an aluminum filter. Output intensities of 125 or 160 kV were employed for 17.5 min to yield 2 and 2.2 kRad irradiation, respectively. Animals were positioned 15 cm from the source and radiation was administered dorsally. Shielding was provided by a 6 mm thickness of lead plate.

Table I

Effect of irradiation on forelimb regeneration

Treatment	Suppressed or aborted[1]	Mid-bud[2]	Palette	Digital
None	0/15	23 ± 4	33 ± 4	42 ± 4
2 kRad whole body (shielded limb)	3/10	22 ± 2	47 ± 12*	53 ± 10*
2 kRad irradiated limb	13/13**	>76**	—	—

[1] Number of cases/total number within group.
[2] Mean number of days ± 1 S.D.
* $P < 0.01$; ** $P < 0.001$.

Skin grafts

Subsequently, one group of nontreated newts and two groups of newts receiving whole-body irradiation received skin allografts. These animals were used to assess effects of irradiation in cellular immunity. Reciprocal allografts consisted of small pieces of skin, approximately 2 mm^2, implanted into wound sites created by removal of skin used as grafts for other animals. Thus each newt served as both a donor and a recipient. In addition, a small group of control and irradiated animals received autografts.

Amputations

The remaining control and irradiated animals were used to evaluate effects on regeneration. All regeneration groups were subjected to unilateral forelimb amputation through the distal stylopodium. In all instances, any portions of humerus extending beyond the wound surface were carefully trimmed.

Results and Discussion

Effects on regeneration

Regeneration occurred among all control animals and progressed to the early digital stage in approximately 42 days (Table I). Whole-body irradiation suppressed regeneration of shielded limbs in 3 of 10 newts and significantly ($P < 0.01$) slowed the rate of regeneration among

Table II

Effect of irradiation on graft rejection

Treatment	Retained skin graft[1]	Interrupted circulation[2]	Loss of pigment	Rejection
Autografts:				
None	3/3	>76	—	—
2 kRad	3/3	>76	—	—
Allografts:				
None	3/14	12 ± 1	18 ± 8	27 ± 7
2 kRad	1/12	18 ± 6*	24 ± 6	33 ± 10
>2 kRad	0/10	19 ± 7*	30 ± 6*	36 ± 6*

[1] Number of cases/total number within group.
[2] Mean number of days ± 1 S.D.
* $P < 0.05$.

the remainder (Table I). Thus, there was an adverse effect on forelimb regeneration of whole-body irradiation.

In contrast to the results of the shielded-limb group, irradiation of limbs caused total suppression of regeneration in all cases. This is in complete accord with observations from other laboratories (reviewed in Wallace, 1981). Moreover, irradiation of the limbs led to severe and persistent inflammation and ultimately to resorption of the limbs in 85% of the cases. These events appeared strikingly reminiscent of those described by Schotté and Butler (1941) in larval *Ambystoma* following limb denervation and by Butler (1933) in larval *Ambystoma* following irradiation. These investigators ascribed the resorption phenomenon to a failure of dedifferentiation to stop and the progressive stages of regeneration to commence. They inferred that apparent resorption of the stump occurred because of an inability to retain an appendage of dedifferentiated tissues. However, the aggravated initial inflammation followed by loss of pigmentation, and subsequent resorption of soft tissues observed in this study resembled more the rejection of a foreign graft (Cohen, 1966). The extent to which this similarity can be pursued and its implications are the objects of additional studies.

Effects on allograft rejection

Skin autografts were tolerated by those few control and irradiated animals used for that purpose (Table II). In addition, rejection of skin allografts occurred in 11 of 14 controls and 21 of 22 irradiated animals (Table II). However, the rate of rejection appeared to be sensitive to irradiation. In particular, irradiation above 2 kRad significantly delayed ($P < 0.05$), but did not suppress, allograft rejection. These results suggest that irradiation affected the immunological status of the newts; however, at the dosages used, this effect did not cripple the newts' immune system.

Demonstration of delays in the rate of regeneration of shielded limbs of otherwise whole-body irradiated animals suggest that one or more factors outside of the amputation site had been adversely affected by irradiation. To this observation, the resorption of amputated irradiated limbs presents an intriguing counterpoint. Furthermore, the persistence of inflammation and subsequent erosion (resorption) of the stump seems reminiscent of graft rejection. Therefore, it is tempting to suggest that the factors extrinsic to the limb that were affected by irradiation are associated with the immune system. In fact, the occurrence of both of these manifestations is consistent with interactions between the immune system and the amputated limb.

Speculations

Similarities between these data and previous observations of limb resorption following irradiation (Butler, 1933) or limb denervation (Schotté and Butler, 1941) in larval *Ambystoma* prompt the speculation: Following amputation, dedifferentiation of local tissues occurs. If dedifferentiated cells are stabilized and activated (*e.g.*, by neurotrophic factors), they modulate immunological expression favoring blastema formation and possibly contributing to promoting blastemal growth. However, if stabilization and activation does not occur, presumptive blastemal cells are eliminated by activated immunological defenses (Prehn, 1970; Coleman *et al.*, 1989). Moreover, the absence of a pool of accumulating blastema cells (*e.g.*, following irradiation) might lead to limb resorption in a futile attempt to establish such a pool.

Conclusions

The design of this investigation does not enable the means through which x-irradiation induced the particular effects observed to be known with certainty; consequently, alternative interpretations of our observations might be equally tenable. Nevertheless, the results of this investigation demonstrate that regeneration of shielded forelimbs by newts otherwise receiving whole-body irradiation occurs in animals that are still immunocompetent, at least in terms of allograft rejection. In addition, these data suggest that x-irradiation can affect the expression of epimorphic regeneration through central, as well as local, effects. Furthermore, this impairment of regeneration appears to occur in parallel to retardation of the rate of allograft rejection. Consequently, a relationship between immunological expression and epimorphic regeneration is suggested. Moreover, these results remove the potentially devastating challenge to this hypothesis presented by earlier investigations of limb regeneration in which x-irradiation was used.

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